Comparison of Denaturing RP-ESI-MS and Native SEC-ESI-MS for the Characterization of Monoclonal Antibodies

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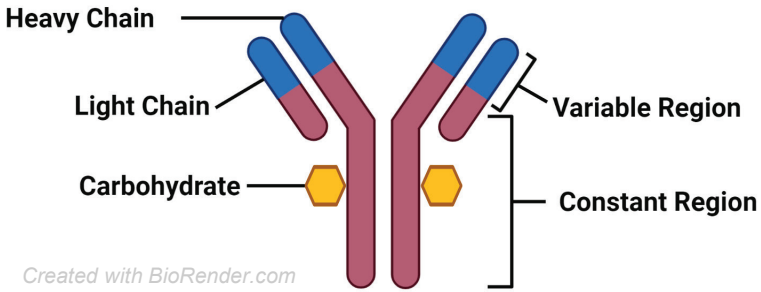
Monoclonal Antibodies are Key Biotherapeutics

- **Therapeutic antibodies treat a plethora of indications** including cancer, infections, autoimmune disorders, and cardiovascular and neurological diseases
- **Therapeutic antibodies are complex, but not too complex…**
  - At 150 kDa it's big, but not huge.
  - The post-translational modifications, such as glycosylation, are not excessive but relatively conserved

HOWEVER:

A lot of the complexity in monoclonal antibodies comes in their characterization (amino acid sequence, glycosylation pattern, C-terminal truncations, degree of aggregation, etc.)

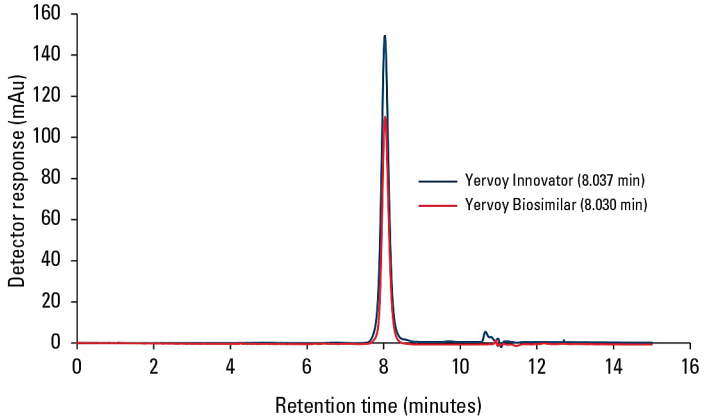
- **Characterization of monoclonal antibodies** (structure, aggregates & impurity levels, post-translational modifications, functions at biomolecular & cellular levels**) is crucial during both the developmental and production phases of the therapeutic antibody**



SEC-UV as the Gold Standard

- Size exclusion chromatography (SEC) is a powerful tool for the separation of biotherapeutics
- Primarily used for the separation in analytical HPLC and for routine quality control analysis of mAbs
- The chromatographic conditions maintain the native structure of the biomolecules
- Conventional SEC analysis of mAbs uses phosphate buffers at pH 6.7

Separation of Yervoy and Biosimilar by TSKgel® UP-SW3000



Column: TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase: 100 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4
Flow rate: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C

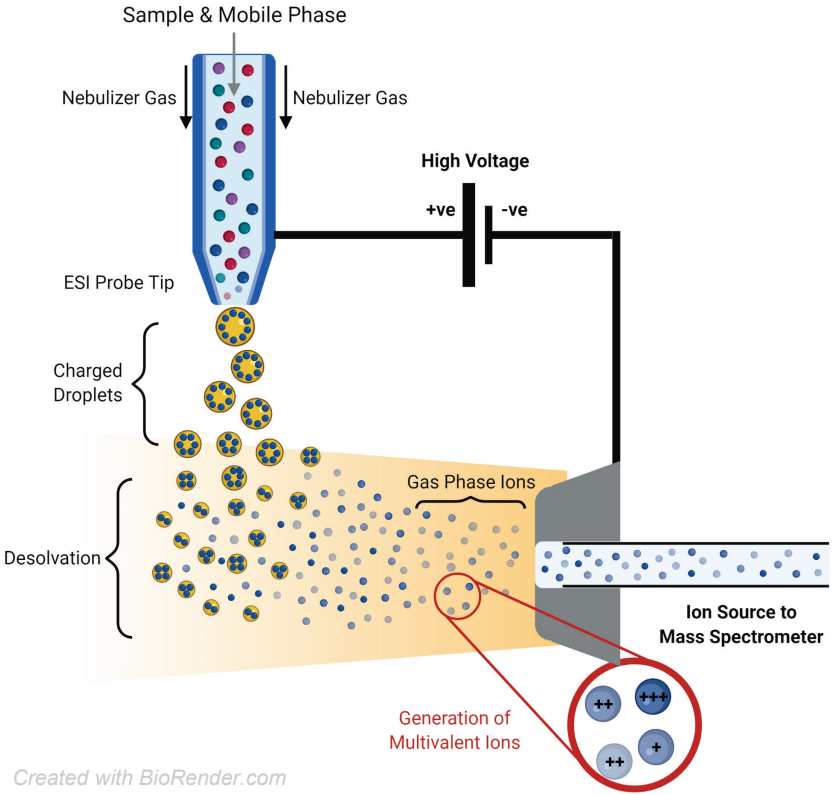
RP-UV is more readily MS-compatible

- With the use of mass spectrometry (MS)-compatible eluents, reversed-phase (RP) chromatography is particularly well suited for protein desalting prior to MS analysis.
- Using a gradient from low to high organic content, the protein binds to the column while MS-interfering contaminants are eluted.
- It is suitable for determining the accurate mass of an intact protein.
- For large proteins, such as monoclonal antibodies, wide pore columns are recommended.
- Additional benefits to RP desalting include the increased mobile phase volatility at the source for an improved ionization process.
- Nevertheless, the use of organic solvent in the mobile phase causes denaturation of the protein, which results in increased charge (z) values and often complicates MS data interpretation.
- In addition, mAb analysis by RP requires high temperatures (usually > 50 ºC) to reduce the strength of secondary interactions between mAb and the stationary phase, which can result in unwanted on-column fragments generation or loss of specific sugars from the glycan moieties.

Why MS Compatibility Matters

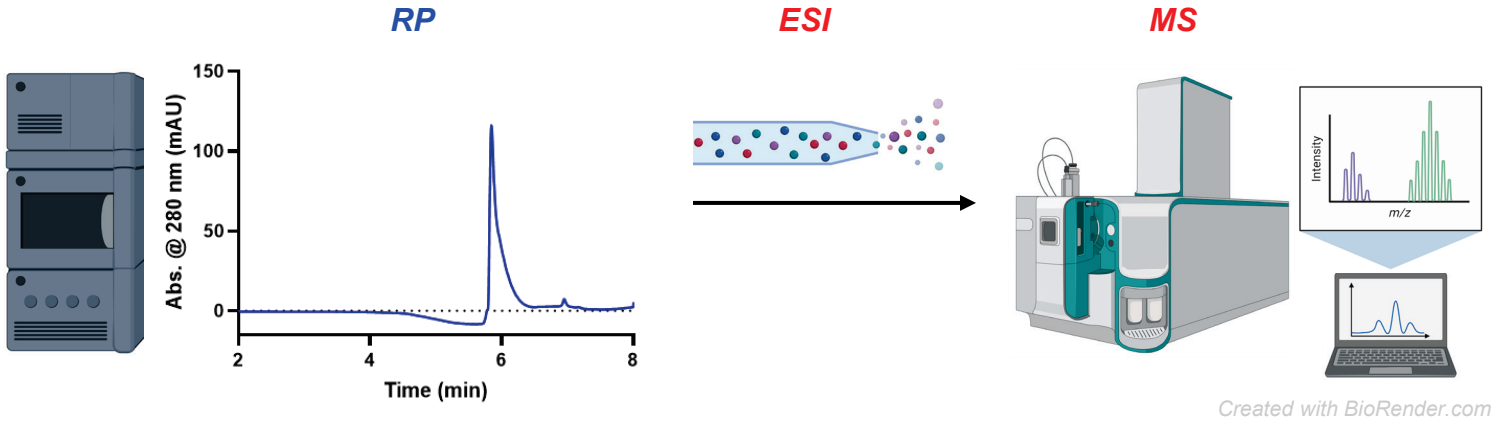
- Most optical detectors (UV, fluorescence) use a flow cell
  - The detection occurs in the liquid phase
- Mass spectrometry detects **ions**
  - MS detection occurs in the gas phase
  - Mobile phase pH and sample pKa information are critical
- LC-UV mobile phases need to be optimized for MS detection
  - Replace non-volatile buffers with volatile buffers
  - The column ID and flow rate should be kept low to increase sensitivity
  - Maintain the pH as in the original separation if possible
- Sample preparation is another key to U/HPLC-UV-MS analysis
  - Eliminate matrix/salt/detergent effects
  - Dilute or diafiltrate the sample in the solvent composition that exists at the start of the LC method

LC-MS Coupling: Ionizing Analytes



- ESI is a “soft ionization” technique, because there is very little fragmentation occurring during ionization, making it very useful for macromolecules analysis.
- ESI is different from other ionization processes because it may produce multiple-charged ions, effectively extending the mass range of the analyzer.
- ESI allows to retain solution-phase information into the gas-phase.
- Electrospray ionization is used to produce ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol.
- Large-flow electrosprays can benefit from nebulization of a heated inert gas (e.g., N_2) to improve desolvation.

RP-ESI-MS – Experimental Conditions

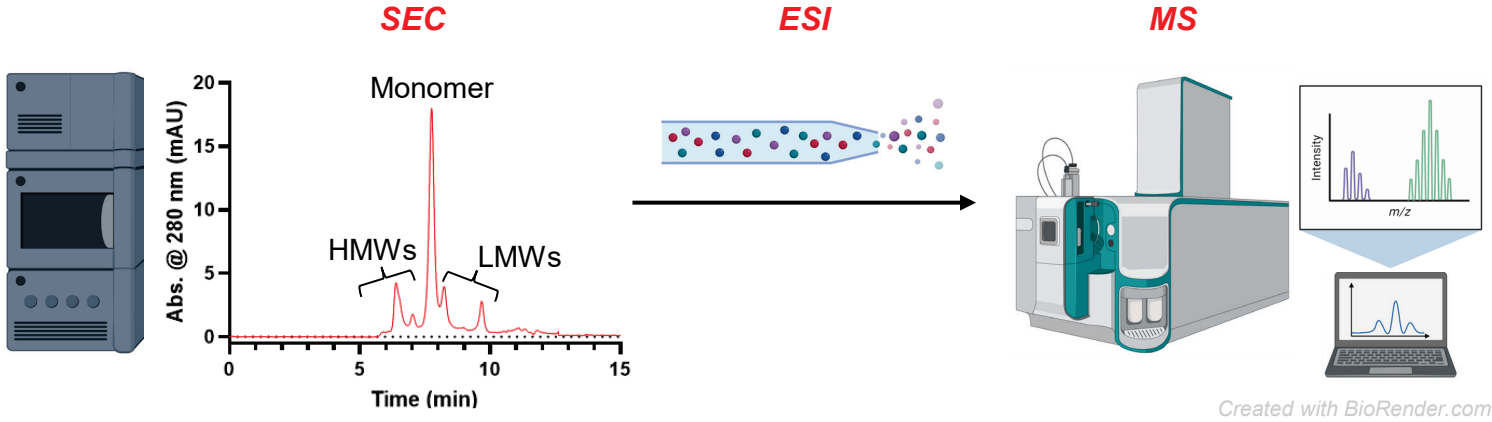


Column:	TSKgel Protein C4-300, 3 µm, 2.0 mm ID × 5 cm
Instrument:	Shimadzu Nexera® XR UHPLC
Mobile phase:	A: water containing 0.1% formic acid (FA) B: acetonitrile (CH_3CN) containing 0.1% FA
Gradient:	0-1 min at 5% B; from 1 (5% B) to 2.5 min (90% B); 2.5-3.5 min at 90% B; Back to 5% B at 3.7 min; 3.7-8 min at 5% B
Flow rate:	0.2 mL/min
Detection:	UV @ 280 nm
Temperature:	40 ºC
Sample Injection:	2 µL of NIST mAb @ 0.5 mg/mL

MS Acquisition on SCIEX X500B QTOF

Ion source gas 1: 45 psi	Declustering potential: 275 V
Ion source gas 2: 45 psi	Collision energy: 20 V
Curtain gas: 30 psi	Accumulation time: 0.5 s
CAD gas: 7 psi	Time bins to sum: 120
Spray voltage: 5000 V	Script of Intact Protein Mode: ON
Source temperature: 450 ºC	Q1 transmission window: 100% at 2250 Da
TOF MS (+) 900-4000 m/z scan	

SEC-ESI-MS – Experimental Conditions



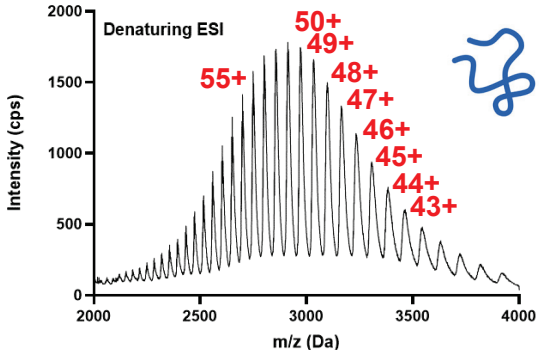
Column:	TSKgel UP-SW300, 2 µm, 4.6 mm ID × 15 cm
Instrument:	Shimadzu Nexera® XR UHPLC
Mobile phase:	50 mmol/L ammonium acetate, pH 6.8
Flow rate:	0.2 mL/min
Detection:	UV @ 280 nm
Temperature:	25 ºC
Sample Injection:	10 µL of NIST mAb @ 0.2 mg/mL

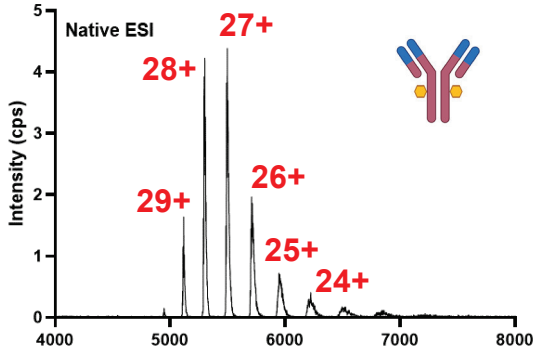
MS Acquisition on SCIEX X500B QTOF

Ion source gas 1: 50 psi	Declustering potential: 275 V
Ion source gas 2: 50 psi	Collision energy: 5 V
Curtain gas: 30 psi	Accumulation time: 0.5 s
CAD gas: 7 psi	Time bins to sum: 80
Spray voltage: 5000 V	Script of Intact Protein Mode: ON
Source temperature: 450 ºC	Q1 transmission window: 100% at 2250 Da
TOF MS (+) 4000-8000 m/z scan	Accumulation time: 1 s

Keeping Intact mAb in its Native State

	Denaturing ESI	Native ESI
Sample Solution	partially organic solution water, formic acid, acetonitrile, methanol (pH 1-2)	aqueous solution water, ammonium acetate (pH 6-9)
Salt Treatment	online/offline desalt with RP-HPLC	offline desalt
Protein Concentration	< 1 µmol/L (subunit)	1-10 µmol/L (complex)
Output Information	MW of single subunit	MW of protein complex noncovalent interactions stoichiometry and structure





Adapting Existing Methods to LC/MS

Buffer considerations for SEC separation

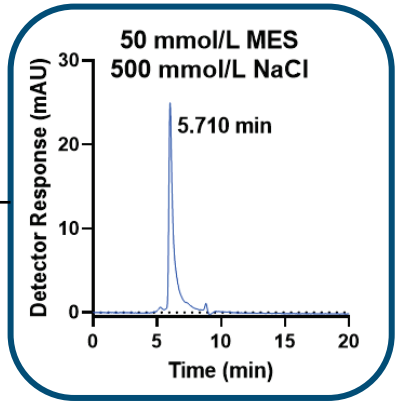
- ***Switching from the "classical" SEC-UV buffers with NaCl to volatile buffers may affect elution profiles***
  - Changes in retention times / Alterations in peak shape
  - Finding the sweet spot: volatile buffer concentration affects SEC elution

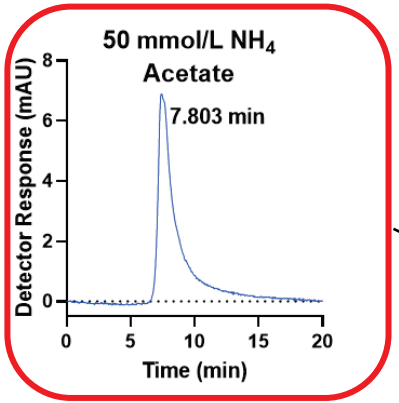
Buffer considerations for MS analysis

- ***Preserving native structures***
  - Protein unfolding during the SEC separation may occur
  - Optimization of volatile salts concentration
- ***Ionization efficiency***

Buffer Considerations for SEC Separation

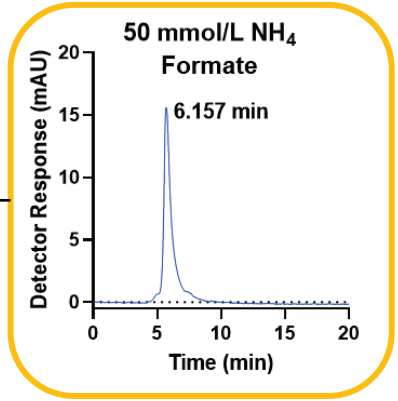
- Sharp peak
- Dirty MS source
- No MS signal

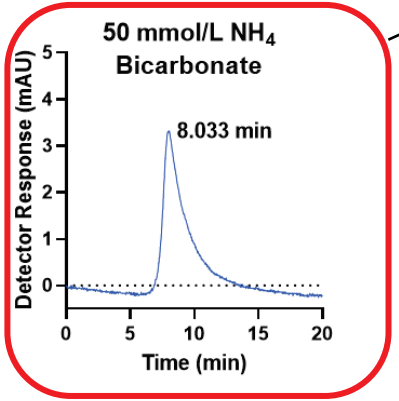




- Broad peak
- Clean MS source
- Good MS signal

- Sharp (*enough*) peak
- Clean MS source
- Good MS signal

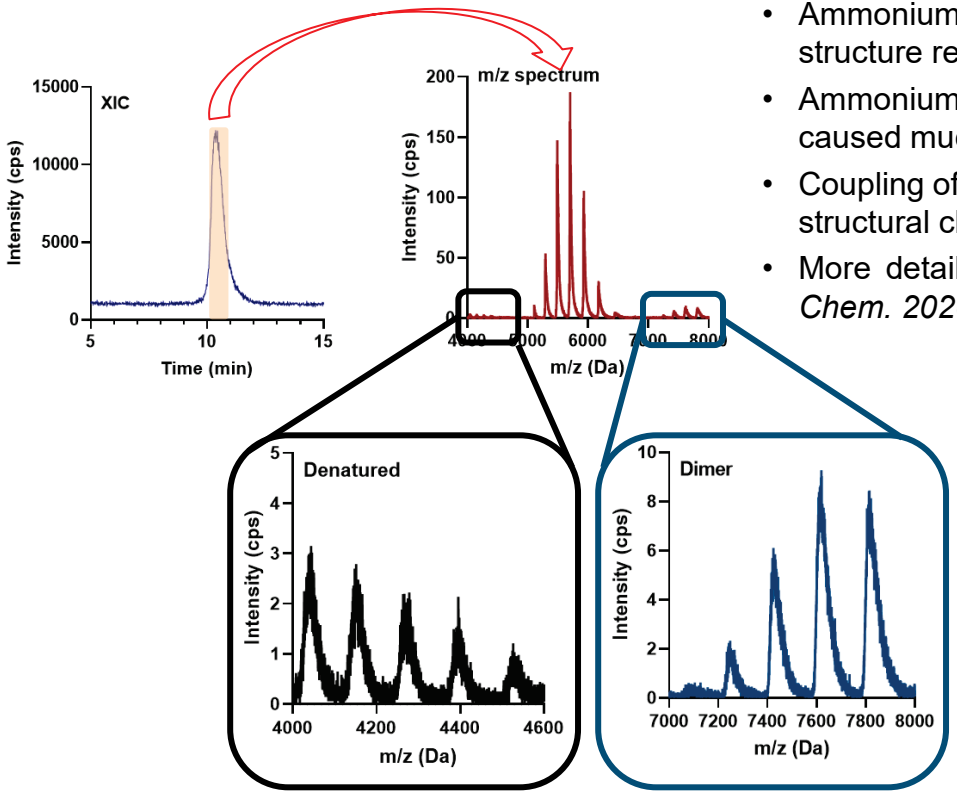




TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm

All mobile phases at pH 6.8
Flow rate: 0.2 mL/min
Detection: UV @ 280 nm
Temperature: 25 ºC
Sample: 1 µg of Humira Biosimilar

Monitoring Native Structures



- Ammonium acetate most effectively preserved the protein structure regardless of ionic strength
- Ammonium formate, and especially ammonium bicarbonate, caused much higher protein denaturation (not shown)
- Coupling of SEC with native ESI-MS enabled monitoring of structural changes during the SEC elution process
- More details on this phenomenon in *IK Ventouri et al. Anal. Chem. 2020, 92 (6) 4292–4300*

TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm

Mobile phase:	50 mmol/L ammonium acetate, pH 6.8
Flow rate:	0.2 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Sample:	2 µg of NIST mAb

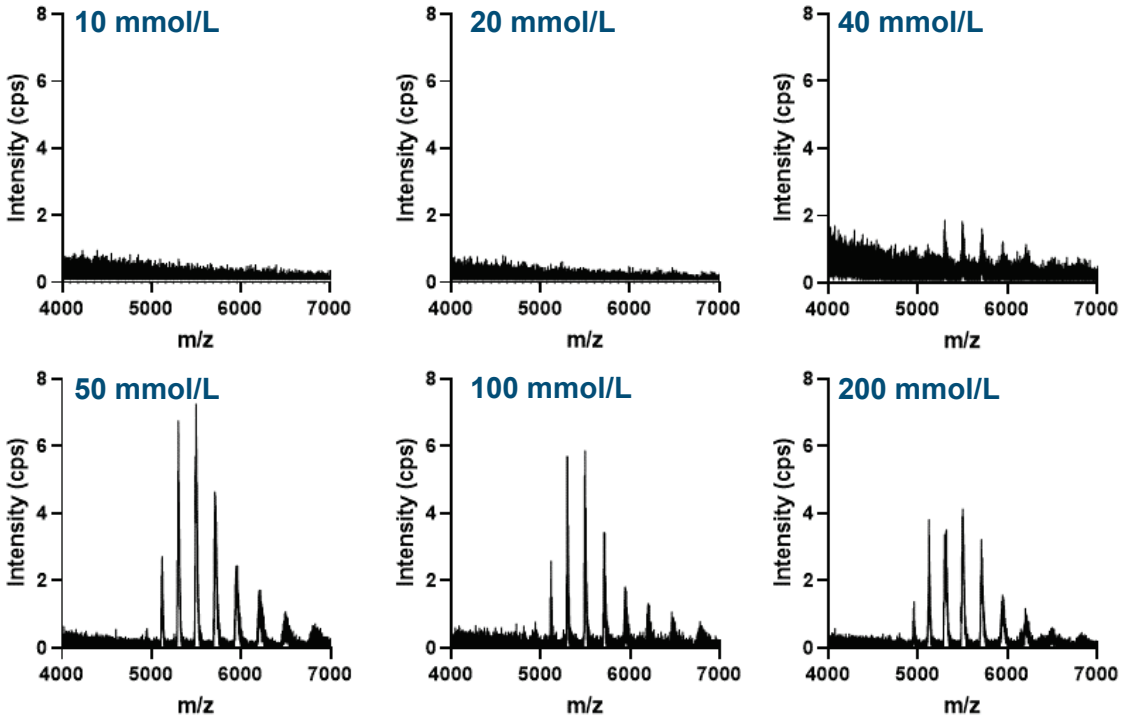
Salt Concentration & Ionization Efficiency

Ammonium Acetate

TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm

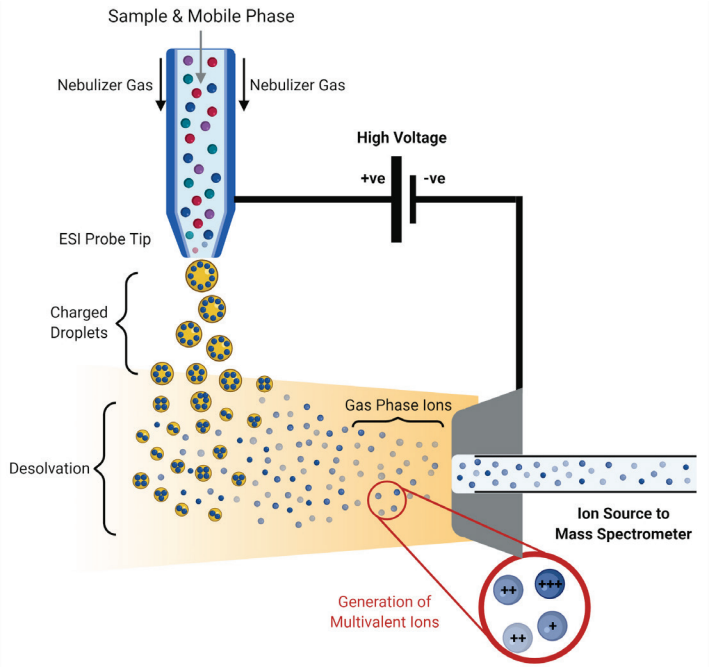
Mobile phase: ammonium acetate, pH 6.8
Flow rate: 0.2 mL/min
Temperature: 25 ºC
Sample: 2 µg NIST mAb

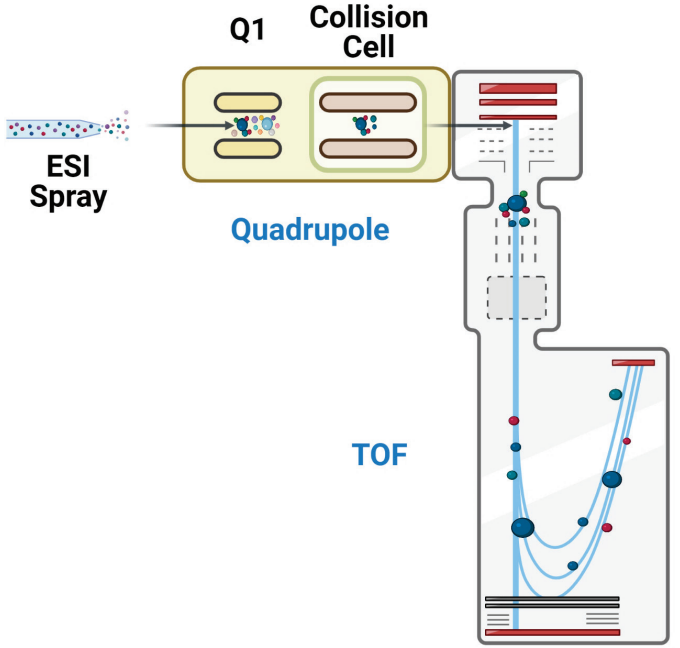
- **In our hands, 50-100 mmol/L ammonium acetate produced the best ionization efficiency.**
- **Needs to be optimized for a given mAb and MS instrument**



Electrospray Ionization efficiency for the NIST mAb separated by SEC using various concentrations of ammonium acetate at pH 6.8

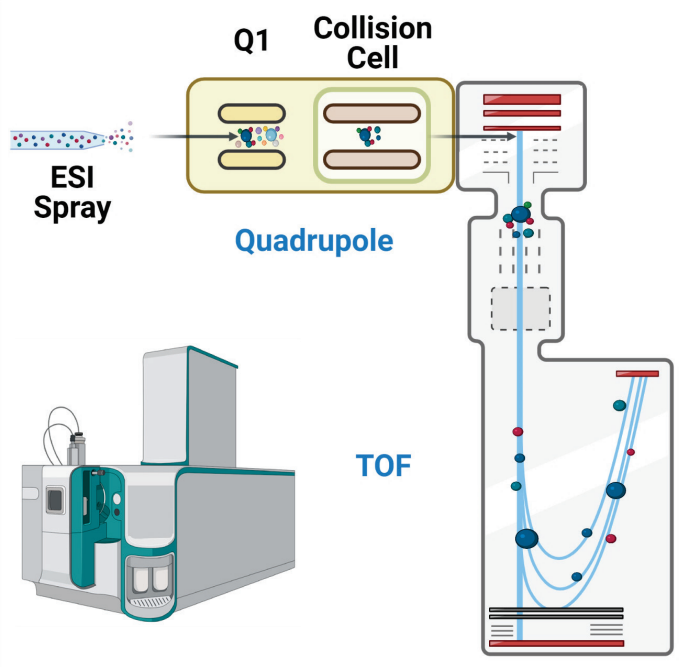
Understanding Your MS Instrument





- **Understanding the inner workings of the MS instrument helps with detection optimization.**
- **There are no details too small with high resolution mass spectrometry.**
- **Once you know your hardware, it is all software!**

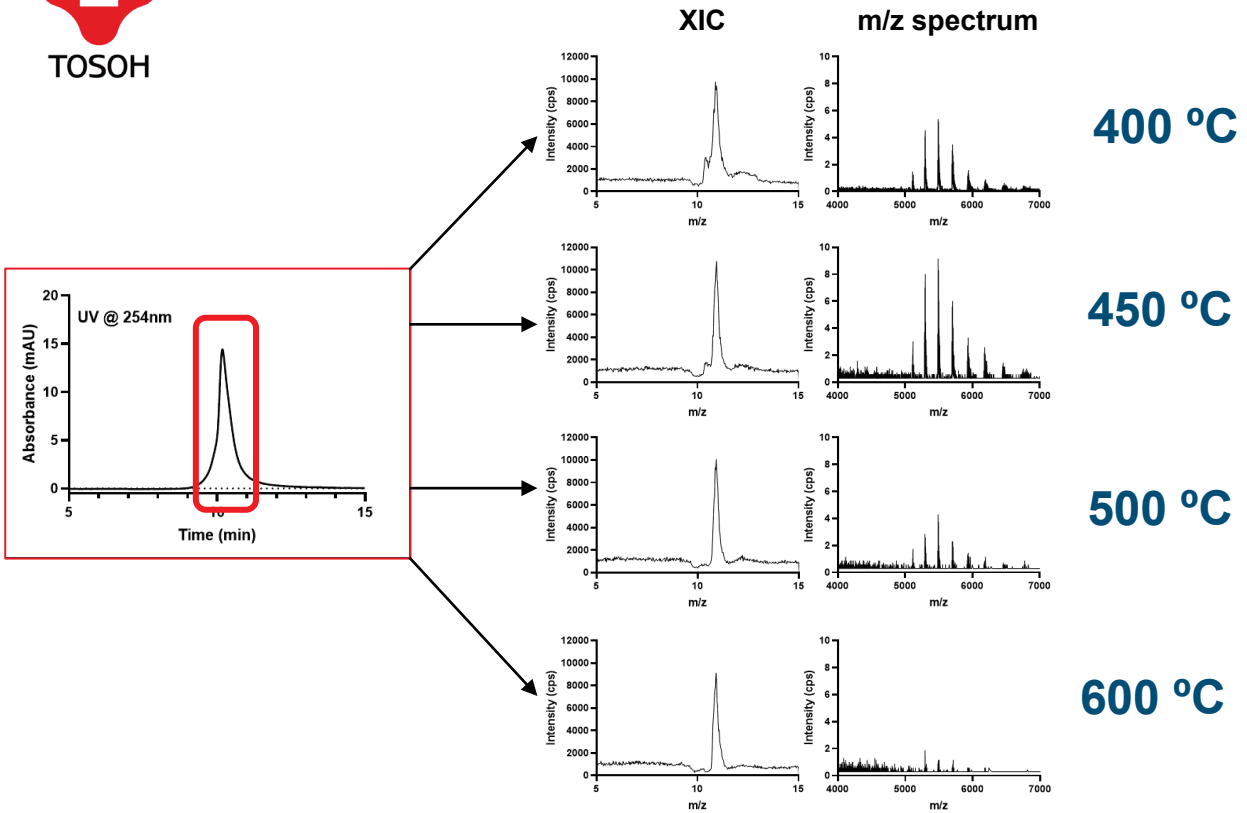
Understanding Your MS Instrument



MS Acquisition on SCIEX X500B QTOF

Ion source gas 1: 50 psi
Ion source gas 2: 50 psi
Curtain gas: 30 psi
CAD gas: 7 psi
Spray voltage: 5000 V
Source temperature: 450 ºC
TOF MS (+) 4000-8000 m/z scan
Declustering potential: 275 V
Collision energy: 5 V
Accumulation time: 0.5 s
Time bins to sum: 80
Script of Intact Protein Mode: ON
Q1 transmission window: 100% at 2250 Da
Accumulation time: 1 s

Source Temperature Effects



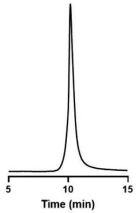
- Compared to small molecules analysis, intact protein analysis requires higher source temperature.
- Usually, the bigger the protein, the higher the temperature.
- It may be mAb-dependent.
- Watch out for protein denaturation!

Tips for Native Protein ESI-MS

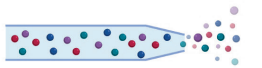
- Optimize the Sprayer Voltage & the Sprayer Position
- Optimize Gas Flow Rates and Temperatures
- Promote Analyte Ionization
- Tune and Calibrate the Instrument
- Select an Appropriate Starting Point –
  and Make Your Way from the Outside In

Optimized SEC-ESI-MS Workflow

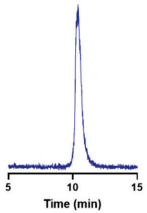
SEC separation on a TSKgel UP-SW column using a U/HPLC system

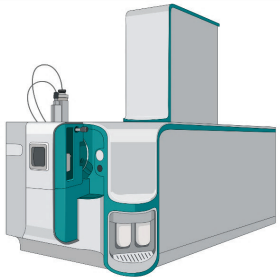


Ionization

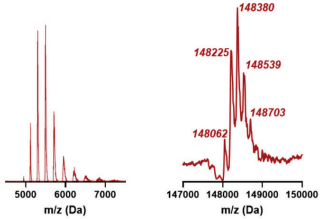


ESI





Native MS Analysis



Buffer	Column
*Type	*Flow Rate
*Concentration	*Column Length
*pH	*Particle Size

Spray voltage: 5000V
Source temperature: 450°C
Ion source gas: 50 psi
Curtain gas: 30 psi

TOF MS (+) 4000-8000 m/z scan
Intact Protein Mode
100% Q1 Transmission >2250 Da
Declustering potential: 275 V
Collision energy: 5 V
Accumulation time: 0.5 s
Time bins to sum: 80
Accumulation time: 1 s

Conclusions

- Monoclonal antibodies are highly complex biomolecules, requiring high resolution, precision and dynamic range, to fully characterize them with confidence.
- This presentation illustrates an intact mAb analysis workflow solution integrating U/HPLC technologies, high-resolution mass spectrometry on a QTOF instrument and software for automated data processing.
- The workflow permits rapid and accurate intact mass characterization of mAbs, using either denaturing ESI using reversed phase chromatography or native ESI using size exclusion chromatography, leading to excellent mass accuracy for glycoform distribution analysis.
- Detailed information was obtained about the heterogeneous composition of mAb proteins, with minimal sample preparation involved.